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**A 39kDa FRAGMENT OF ENDOGENOUS ASK1 SUGGESTS SPECIFIC
CLEAVAGE NOT DEGRADATION BY THE PROTEASOME**

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Abstract

Transfected human ASK1 produces a 150kDa protein. However, we have detected endogenous ASK1 predominantly as 39kDa and 50kDa C-terminal and 75kDa and 110kDa N-terminal fragments in a panel of non-transfected cancer cell lines and HUVEC endothelial cells. This suggests that in non-apoptotic cells, endogenous ASK1 protein is normally cleaved at a number of specific sites, some of which are in the kinase domain. Transfected ASK1 protein is known to be degraded by the proteasome. In contrast, the cleavage of endogenous ASK1 is independent of the proteasome as treatment with the proteasome inhibitor, lactacystin did not inhibit cleavage. Cisplatin treatment decreased the amount of 39kDa C-terminal ASK1 fragment in mutant p53 cell lines suggesting a decrease in cleavage associated with apoptosis. Transfected ASK1 may therefore not accurately reflect the role of endogenous ASK1.

Introduction

ASK1 (Apoptosis Signal-Regulating Kinase 1) is a member of the mitogen activated protein (MAP) kinase family of proteins and is involved in the regulation of apoptosis. ASK1 is endogenously expressed at low levels so the vast majority of experimental work characterising ASK1 has been performed in transfected cell lines. The human ASK1 protein consists of 1375 amino acids with a serine/threonine kinase domain in the middle of the molecule (1). The N and C-terminals of ASK1 are coiled-coil domains (1) to which regulatory molecules such as thioredoxin and glutaredoxin bind respectively to inhibit ASK1 kinase activity (2). ASK1 has been shown to be regulated by ubiquitination, inactive ASK1 with bound thioredoxin is a target for degradation by the proteasome (3).

The majority of studies of ASK1's regulation and interaction with the apoptotic pathways has been performed in cell lines transfected with the pcDNA3-ASK1-HA plasmid (4). While these studies are useful in elucidating the function of ASK1 and its role in the apoptotic pathways, such artificially high levels of ASK1 may produce different results to endogenous levels of ASK1. We have examined the endogenous expression of ASK1 in a panel of non-transfected cell lines and have found that ASK1 is cleaved into two major fragments which may represent a novel mechanism of inactivation in non-apoptotic cells.

Methods

Cell Culture

The human cancer cell lines H69, CEM, U87-MG were obtained from the ATCC (Virginia, USA). HUVEC cells were obtained by primary culture. H69 and CEM cells were grown in antibiotic-free RPMI (Thermoelectron, Sydney, Australia) with 10% Foetal Calf Serum (FCS) (Thermoelectron). U87-MG cells were grown in antibiotic-free D-MEM/F-12 (Gibco via Invitrogen, Melbourne, Australia) with 20% FCS. HUVEC cells were grown in D-MEM/F-12 with 20% FCS, 50µg/ml heparin, 50µg/ml endothelial cell growth supplement and 40µg/ml gentamycin. All cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. Cultures were *mycoplasma* free. The suspension cell lines (H69 and CEM) were treated with cisplatin, MG132, lactacystin or leupeptin at a cell density of 5.0×10^5 /ml. The attached cell lines (U87-MG and HUVEC) were seeded for drug treatment such that the control flasks would reach confluence after 48 hours.

Transfection of ASK1 into H69 cells

The pcDNA3-ASK1-HA plasmid was a gift from Dr. Hidenori Ichijo (4). Plasmids were purified from JM109 cells using a Qiagen (Melbourne, Australia) Endofree Plasmid Maxi Kit. 1.3×10^6 H69 cells were resuspended in 1.6ml RPMI with serum and 0.1mg/ml neomycin in a 6 well plate. 0.8µg plasmid DNA in 100µl Qiagen Buffer EC was

combined with 6.4µl Qiagen Enhancer and 40µl Qiagen Effectene transfection reagent and incubated for 10 minutes at room temperature. 600µl RPMI was added and the transfection mix added to the cells.

Western Blotting

Cells were washed in cold PBS (0.15M NaCl, 0.03M NaH₂PO₄, 0.07M Na₂HPO₄, pH 7.2), and resuspended in 100µl of lysis buffer (0.01M Tris/HCl, pH 7.4) at 4°C. 10µl complete protease inhibitor (Roche, Sydney, Australia) was added prior to sonication. 20µg protein was then electrophoresed and Western blotted as previously described (5), with the following modifications. A 12% acrylamide Tris/glycine gels with a 4% stacking gel was used and Biorad markers or Biorad broad range markers were used as indicated (Biorad, Sydney, Australia). Primary antibodies used were anti-ASK1 C-terminal epitope 1275-1290 (Upstate, Melbourne, Australia). Anti-ASK1 C-terminal epitope 1076-1375 (Santa Cruz Biotechnology via Monarch Medical, Brisbane, Australia). Anti-ASK1 N-terminal epitope 20 amino acids surrounding 280 (Cell Signaling Technology, via Genesearch, Brisbane, Australia). The ASK1 antibodies were used at a dilution of 1:500, 1:200 and 1:500 respectively. The anti ASK1 C-terminal inhibition peptide, the epitope of the antibody (EEKDQEIKHLKLKSQP-NH₂), was incubated with the primary antibody at a ratio of 5:1 (Upstate). The p21^{WAF1} antibody (Clone CP74 from Labvision), was used at a 1:1000 dilution. The secondary antibody used was an alkaline phosphatase conjugated rabbit immunoglobulin (Chemicon, Melbourne, Australia), diluted 1:500.

Results

Endogenous ASK1 is cleaved

ASK1 was examined by Western blot using a C-terminal ASK1 antibody in H69, CEM, and U87-MG cancer cell lines and in HUVEC endothelial cells (Figure 1A). This revealed a prominent 39kDa band in all cell lines, a 50kDa band and some multi-banding in the 150kDa range, the size of full-length ASK1, in the U87-MG and HUVEC cells. A low molecular weight fragment of ASK1 has not been previously reported in the literature so we investigated the specificity of the antibody. The major 39kDa and 50kDa bands as well as the higher molecular weight minor bands were absent on incubation with an inhibition peptide, the epitope of the antibody (Figure 1B). A blast search of human protein sequences also confirmed that the C-terminal antibody's epitope was specific to human ASK1 (6). Another C-terminal ASK1 antibody (Santa Cruz Biotechnology) was tested on the H69 cells and the same 39kDa band was detected (data not shown), suggesting that the 39kDa band was not an artefact of individual antibody.

An N-terminal ASK1 antibody detected 2 prominent bands both smaller than 150kDa (Figure 1C). The molecular weights of the C and N-terminal fragments were calculated and averaged from at least 5 independent Western blots using 2 different molecular weight markers for quantitation (Figure 1D). When the size of the major and minor bands were added from both the C and N-terminal, the total approaches the molecular weight of full-length ASK1. Figure 1E suggests potential cleavage sites determined by the size of

the fragments using the data from Figure 1D using the protein size calculator Protcalc (7). This suggests that there are several cleavage sites which may cleave and inactivate the kinase domain or alter the normal regulation of kinase activity. The C-terminal of the protein is cleaved away into the 39kDa fragment and the kinase domain may also be cleaved forming the 50kDa fragment.

As a control the pcDNA3-ASK1-HA plasmid (4) was transfected into H69 cells to test if full-length ASK1 could be detected using the C-terminal ASK1 (upstate) antibody (Figure 1F). The 39kDa band is present in all samples, the 150kDa band is present in the ASK1 transfected sample 72 hours after transfection and not in any of the transfection controls. This indicates that the C-terminal ASK1 antibody is capable of detecting full-length ASK1 when it is overexpressed.

Modulation of ASK1 cleavage products in response to cisplatin

Cells were treated with 5 μ g/ml cisplatin for 48 hours, a dose high enough to trigger cell cycle arrest and apoptosis. The amount of the 39kDa C-terminal ASK1 band decreased in response to cisplatin treatment in the H69 and CEM cells which have mutant p53 (Figure 2A). Some faint bands were also observed in the ~150kDa region in response to cisplatin treatment suggesting that some full-length ASK1 may be present in the apoptotic samples. Earlier and later time points were also examined, however, no clear 150kDa ASK1 band was detected. There was no change in amount of the 39 or 50kDa bands in wild type p53 cells U87-MG and HUVEC.

Is the proteasome responsible for ASK1 cleavage?

ASK1 is inactivated by ubiquitination and degradation in cell lines which have been transfected with ASK1 (3). As the 39kDa band was so sharp we theorised that it was the product of a specific cleavage rather than degradation by the proteasome. H69 cells were treated with 10 μ M MG132 a reversible proteasome inhibitor for 24 and 48 hours. As a positive control for proteasome inhibition the accumulation of cell cycle protein p21 was examined (Figure 3A). The 48-hour MG132 treatment caused an accumulation of p21 suggesting successful proteasome inhibition and the C-terminal ASK1 fragment disappeared. This suggested that the proteasome may be involved in the degradation of endogenous ASK1. However, MG132 is also known to inhibit certain lysosomal cysteine proteases and calpains and have apoptotic activity (8). Therefore another proteasome inhibitor lactacystin was used to confirm the involvement of the proteasome. Leupeptin which inhibits the calpains, trypsin and cathepsins (9) was also used to examine the role of these proteases. H69 cells were treated with 10 μ M lactacystin or 8 μ g/ml leupeptin for 48 hours (Figure 3B). No reversal of ASK1 cleavage was observed suggesting that the proteasome, calpains, trypsin and cathepsins are not involved in ASK1 cleavage.

Discussion

Why have these ASK1 fragments not been previously reported?

Although full-length 150kDa ASK1 was easily detected in ASK1-transfected cells by an antibody to the C-terminal of ASK1, it was not detected in the non-transfected cells. Instead, all cells had a prominent sharp band at 39kDa suggesting that endogenous ASK1 may be specifically cleaved. These bands occurred in a panel of cancer cell lines H69, CEM and U87-MG as well as in HUVEC endothelial cells suggesting that this is not just a cancer specific phenomenon. There are no reports of such cleavage of transfected ASK1. This may be because the majority of studies which have transfected ASK1 into cells using the pcDNA3-ASK1-HA plasmid have also used an HA antibody as a detection system rather than an ASK1 antibody, therefore not detecting any endogenous ASK1. Most publications also only show a narrow profile of the full-length ASK1 150kDa region and not the entire length of the lane where cleavage products could be observed. The cleavage products of endogenous ASK1 may have been previously ignored as when ASK1 is transfected into cells, the 150kDa product becomes so intense that lower molecular weight bands may have been attributed to non-specific reactions.

A role for cleaved endogenous ASK1

Inactivation by ubiquitination and degradation has been well documented for transfected ASK1 (3), this process would not produce cleavage products to detect. It is possible that

ubiquitination and degradation is not as important in the regulation of endogenous ASK1 and that other mechanisms of degradation such as inactivation by cleavage can regulate the lower levels of ASK1 normally expressed. These large fragments of ASK1 may have a biological function as studies of transfection of truncated ASK1 have shown that thioredoxin and glutaredoxin still bind to the N and C-terminals and that that some truncated ASK1 proteins actually have increased kinase activity due to the loss of regulatory domains (10,11).

There is a different pattern of ASK1 fragments which correlates with the p53 status of the cell line (Figure 2). The p53 mutant cell lines H69 and CEM have the 39kDa C-terminal band, whereas the p53 wild type cells U87-MG, HUVEC have both the 39kDa and 50kDa C-terminal bands. The response of the cleavage products to cisplatin treatment is also different between p53 mutant and wild type cells. The wild type cells show no change in the amount of either bands. In contrast, the p53 mutant cells show a decrease in the 39kDa band, suggesting a decrease in ASK1 cleavage resulting in an increase in full-length ASK1 in the apoptotic cells.

Endogenous ASK1 is not cleaved by the proteasome

The proteasome is not responsible for ASK1 cleavage as lactacystin did not reverse the cleavage. The proteasome is also unlikely to be involved as its function is to degrade proteins into small peptides of 3-20 residues not produce large protein fragments which could be biologically active (8). ASK1 is either cleaved by a protease which is inhibited

by MG132 (Figure 3) or MG132 is having an apoptotic effect and reversing the cleavage in a similar manner to the treatment with cisplatin (Figure 2).

Conclusions

Endogenous ASK1 is normally cleaved in cells, this cleavage is specific and may inactivate the kinase domain of the protein. The degradation of endogenous ASK1 is independent of the proteasome which is different to the degradation characterised for transfected ASK1. Transfected ASK1 may therefore not accurately model endogenous ASK1.

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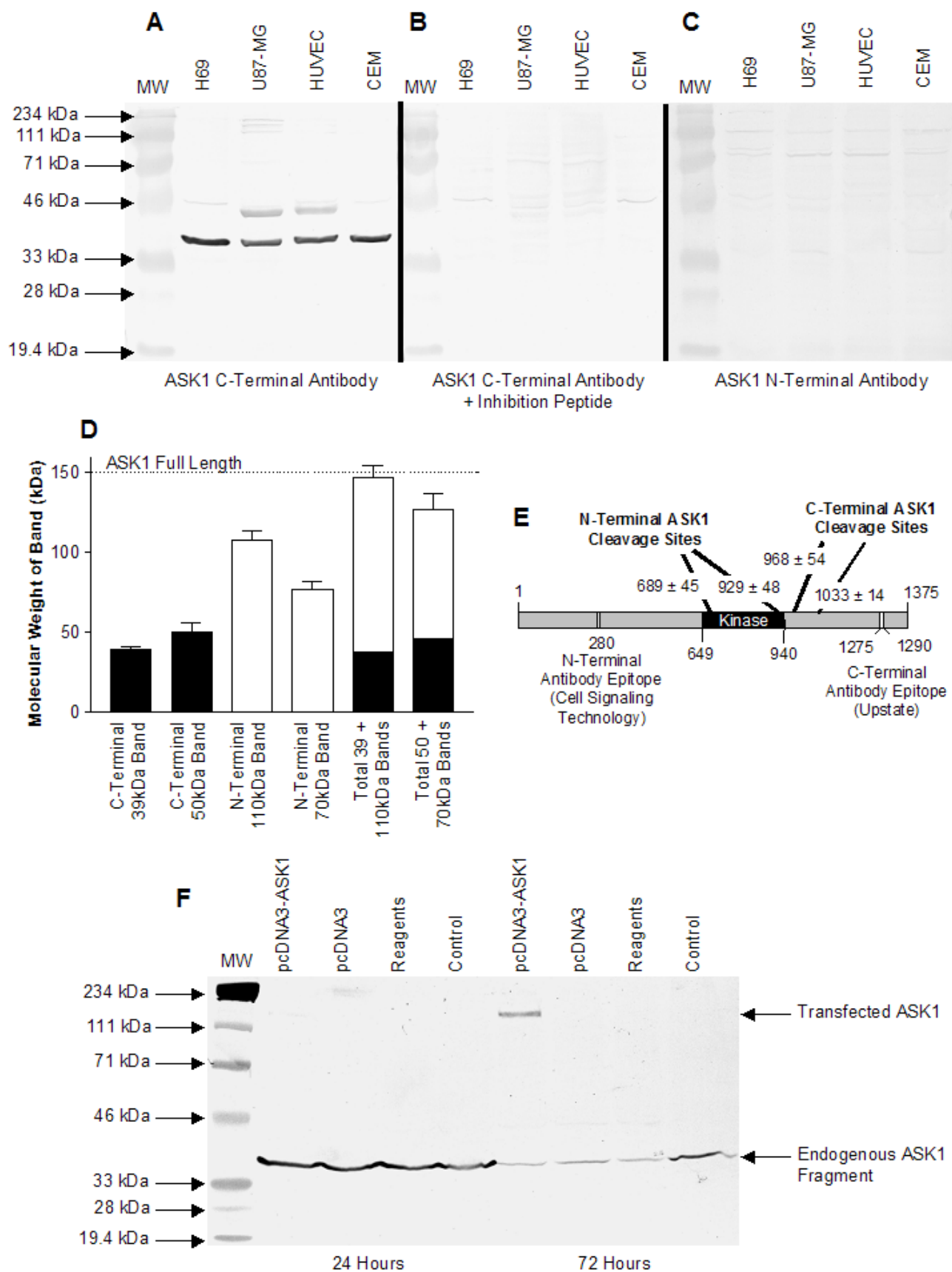


Figure 1 – Analysis of ASK1 cleavage products. Protein from H69, U87-MG, HUVEC and CEM cells was analysed by Western blot with A) ASK1 C-terminal Antibody, B) ASK1 C-terminal Antibody with inhibition peptide and C) ASK1 N-terminal Antibody. D) Molecular weights of ASK1 cleavage products. E) Potential cleavage sites of the ASK1 protein based on the molecular weights of cleavage products. F) Positive control transfection of ASK1. H69 cells were transfected with the pcDNA3-ASK1-HA plasmid and the C-terminal of ASK1 was analysed by Western Blot. Cells were analysed 24 and 72 hours post transfection.

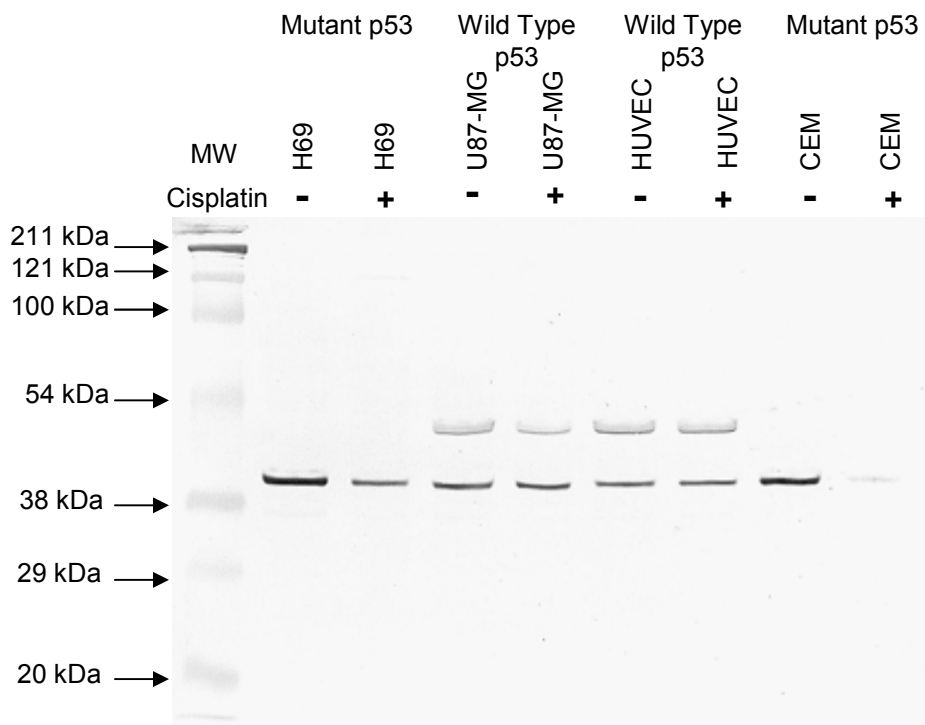


Figure 2 – Modulation of ASK1 cleavage products by cisplatin.
A) H69, U87-MG, HUVEC and CEM cells were treated $\pm 5\mu\text{g/ml}$ cisplatin for 48 hours, and the C-terminal fragments of ASK1 analysed by Western Blot. The p53 status of each cell line is indicated.

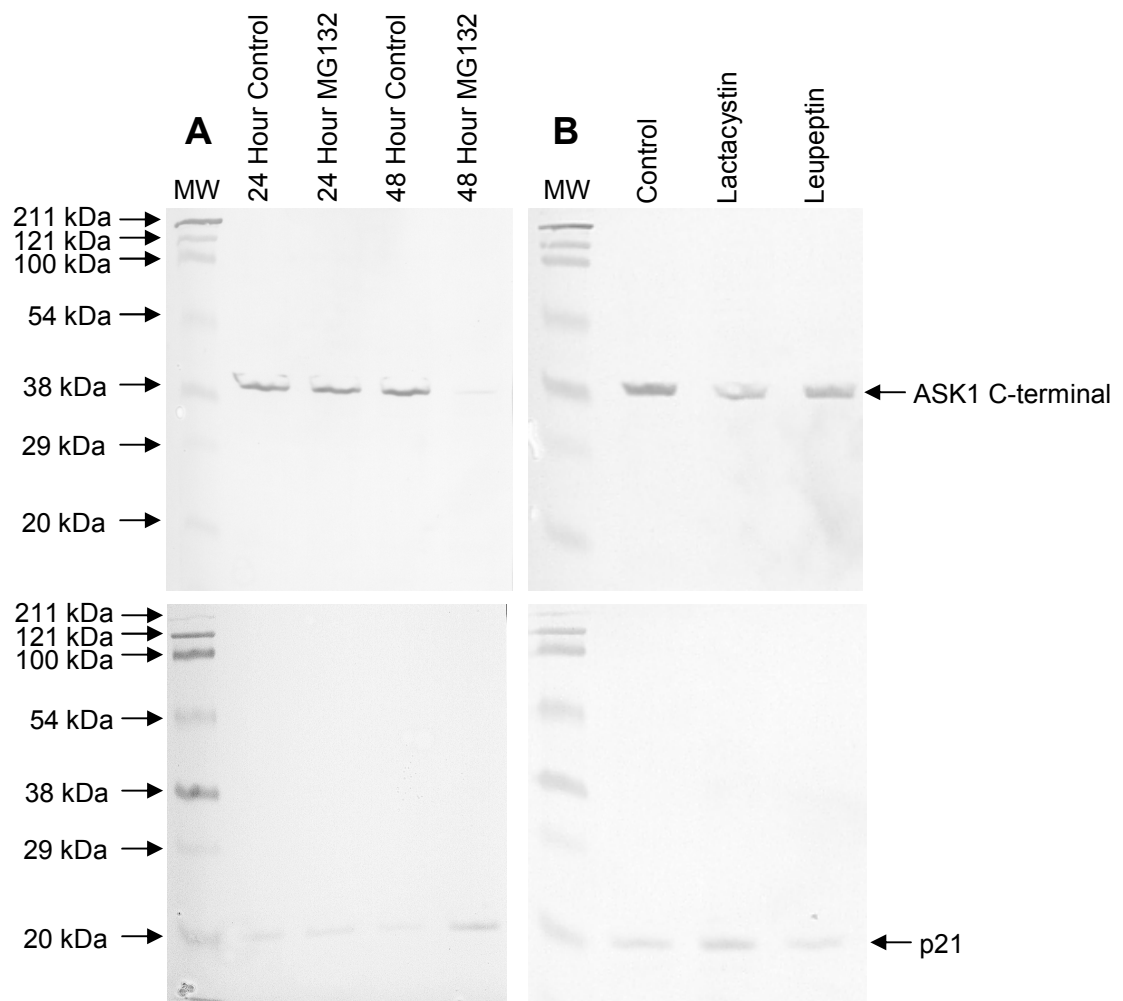


Figure 3 – Inhibition of ASK1 degradation with specific inhibitors. H69 cells were treated A) \pm 10 μ M MG132 and B) \pm 10 μ M lactacystin or 8 μ g/ml leupeptin for 48 hours. The C-terminal fragments of ASK1 and p21 were analysed by Western blot.